VOL. 54 NO. 9, SEPT. 2001 THE JOURNAL OF ANTIBIOTICS pp. 737 — 743

A Novel Assay for Fungal Ketol-isomerase Activity

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(Received for publication May 1, 2001)

2-Deoxy-D-glucose-6-phosphate ketol-isomerase (EC 2.6.1.16) forms glucosamine-6-phosphate and glutamate from fructose-6-phosphate and glutamine and plays an important role in chitin synthesis in fungi. We have established a new assay for fungal ketol-isomerase activity that is amenable to high throughput screening to identify enzyme inhibitors. *Aspergillus fumigatus* crude lysate was incubated with substrates and after incubation, reactions were terminated. Glutamate dehydrogenase, nitro blue tetrazolium chloride, phenazine methosulfate and β-NAD were added and the amount of glutamate formed by ketol-isomerase activity was determined by measuring OD_{585nm}. A feedback inhibitor, UDP-*N*-acetylglucosamine, of fungal ketol-isomerase was successfully detected by this assay (IC_{50}=0.48 mM). In a pilot scale screening, an active extract from an extremophilic bacterium was found, and the extract showed antifungal activity against *A. fumigatus*, *Candida albicans* and *C. glabrata*.

During the last three decades, there has been a dramatic increase in the frequency of fungal infections, especially disseminated systemic mycoses in immunocompromised patients[1,2]. Human pathogenic fungi of particular importance include: *Candida* spp. (*e.g.*, *C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis*), *Aspergillus fumigatus* and *Cryptococcus neoformans*. *C. albicans* and *A. fumigatus* cause most opportunistic mycoses. At present, treatments for fungal infections are limited to few options. Amphotericin B (a polyene) shows a fungicidal effect, but is toxic to humans[3]. Azoles, such as fluconazole and itraconazole, are safer than amphotericin B, but are fungistatic[3]. In addition, resistance to azoles has become an increasing problem in the treatment of fungal infections[4]. Thus, there remains a need for the development of new drugs useful for treatment of fungal infections.

The most striking difference between fungal cells and human cells is that fungal cells are encased in a wall that protects them from an osmotically and immunologically hostile external environment. The cell wall of fungi has a complex composition and structure. Yeast cell walls consist of roughly equimolar amounts of (1,3)β-glucan, (1,6)β-glucan, mannoprotein, and submolar amounts of chitin[5]. Chitin, although a minor component of yeast and filamentous fungal cell walls, is essential for cell viability and mother-daughter cell separation[6,7]. Therefore, chitin synthase has been considered a possible target for antifungal treatment[8~11]. Unfortunately, although polyoxins and nikkomycins inhibit chitin synthase and the growth of fungi[12], none of them has proven useful as therapeutic agents.

Chitin synthases polymerize UDP-*N*-acetylglucosamine (UDP-GlcNAc) to a (1,4)β-linked polymer of *N*-acetylglucosamine, chitin. The precursor, UDP-GlcNAc, is formed from fructose-6-phosphate *via* four sequential enzymatic reactions—the Leloir Pathway[13]. The first enzyme of the Leloir Pathway is 2-deoxy-D-glucose-6-phosphate ketol-isomerase (ketol-isomerase) (EC 2.6.1.16), which is variously called: glutamine: fructose-6-phosphate amidotransferase or glucosamine-6-phosphate synthase. This enzyme forms glucosamine-6-phosphate (GlcN-6-P) and glutamate from fructose-6-phosphate and glutamine[14].

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Ketol-isomerase activity, as well as chitin synthase activity, plays an important role in germ tube formation in *C. albicans*. Fungal ketol-isomerases are feedback-inhibited by UDP-GlcNAc, an end product of the Leloir pathway[15,16]. Accordingly, it is likely that ketol-isomerase activity regulates chitin/hexosamine synthesis. Mutations that destroy ketol-isomerase activity result in the inhibition of fungal growth due to a reduced amount of chitin in cell walls, leading to osmotic sensitivity and fungal cell lysis[17,18]. Importantly, compounds that inhibit fungal ketol-isomerase activity also inhibit *C. albicans* growth[19]. Taken together, ketol-isomerase is thought to be an unexploited target for antifungal agents.

In this paper, we describe a new *in vitro* assay for ketol-isomerase activity using phenazine methosulfate and nitro blue tetrazolium chloride that is suitable for large scale screening of natural products and chemical compounds. In addition, we present the results of a pilot scale screening of natural products for ketol-isomerase inhibitors.

Material and Methods

Chemicals

L-Glutamine was purchased from ICN (Aurora, OH). Bio-Rad protein assay reagent was purchased from Bio-Rad (Hercules, CA). D-Fructose-6-phosphate (Fru-6-P), nitro blue tetrazolium chloride (nitro BT), phenazine methosulfate (PMS), β-NAD and glutamate dehydrogenase Type I from bovine liver (GLDH) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade and distilled, deionized water was used.

Growth and Harvesting of *A. fumigatus*

A. fumigatus ATCC 16424, a human clinical isolate, was obtained from the American Type Culture Collection (Rockville, MD). Frozen stock conidia were thawed at room temperature, harvested, inoculated into YG medium containing 1% (w/v) yeast extract and 1% (w/v) glucose with 1×10^7 conidia per/ml (final concentration), and incubated for 20 hours at 37°C with shaking (250 rpm). Hyphae were harvested by vacuum filtration over Whatman No. 2 filter paper, washed twice with ice-cold water, quick-frozen in dry ice, and stored at −80°C until used.

Preparation of Crude Cell Lysates

A. fumigatus hyphae were thawed on ice and resuspended in KI buffer (600 mM sucrose, 1 mM KCl, 1 mM EDTA, 50 mM PIPES, pH 6.8). Hyphae were disrupted by bead-beating (6×30 seconds pulses with 2 minutes cooling between each pulse) with 0.5 mm zirconium beads using a bead beater (Biospec Products, Bartlesville, OK). The lysates were centrifuged at 1,000 *g* for 10 minutes at 4°C. The supernatants were stored at −80°C until used. Prior to use, each cell lysate was thawed, and the protein concentration was adjusted to 0.3~0.4 mg/ml with KI buffer.

Assay for Ketol-isomerase Activity (Nitro BT Assay)

An *in vitro* assay of ketol-isomerase activity was performed in flat-bottom 96-well microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ). The assay mixtures containing 10 mM Fru-6-P, 10 mM glutamine and 0.2~0.3 mg/ml cell lysate in a final volume of 50 μl were incubated at 37°C for 60 minutes (unless stated otherwise). The reactions were terminated by heating at 70°C for 15 minutes. Then, 50 μl of GLDH solution containing 2 mM β-NAD, 16 μM PMS, 1 mM nitro BT, and 5 units/ml GLDH in 100 mM potassium phosphate buffer (pH 8.2) was added. After incubation at 37°C for 60 minutes, the optical density (OD) at 585 nm was measured using a plate reader (Spectra MAX 340, Molecular Devices, Sunnyvale, CA). Glutamate standards in cell lysate were determined in parallel to generate a standard curve. The amounts of glutamate formed by ketol-isomerase activity were calculated from the standard curve.

To test the effect of putative inhibitors, 2 μl of each sample dissolved in DMSO were added before addition of cell lysate. The assay was performed as described above.

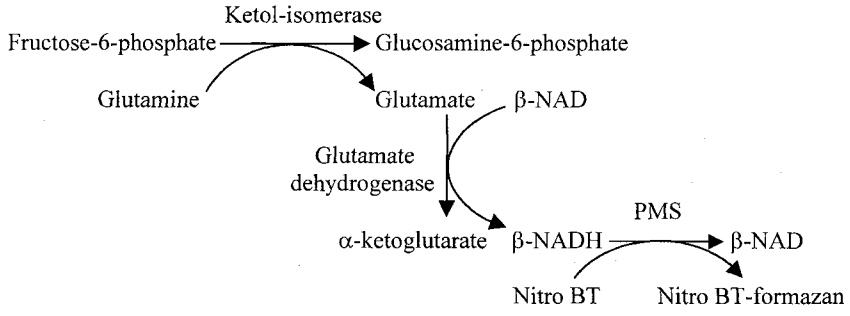
Comparison between Glutamate and GlcN-6-P Concentrations

Ketol-isomerase reactions were performed as described above except that the volume was 100 μl in micro-centrifuge tubes and reactions were terminated by boiling for 5 minutes. After termination, precipitated protein was removed by centrifugation at 10,000 *g* for 10 minutes. The amounts of glutamate and GlcN-6-P formed by reactions were determined by the nitro BT assay and the Morgan-Elson assay (described below), respectively.

Morgan-Elson Assay

The amounts of GlcN-6-P formed by reaction mixtures were determined by using a modification of the Morgan-Elson assay[20]. Briefly, 5 μl of saturated $NaHCO_3$ and 5 μl of cold 5% acetic anhydride were added to 30 μl aliquots of reaction mixtures (prepared and incubated as described above) in micro-centrifuge tubes. After incubation at room temperature for 3 minutes, tubes were boiled for 3 minutes, and then 50 μl of 0.27 M sodium borate was added. The

Fig. 1. The experimental design for a new ketol-isomerase assay.



tubes were boiled for 8 minutes, and cooled to room temperature, and 400 μl of Ehrlich's reagent was added. Tubes were incubated at 37°C for 20 minutes and then the OD_{585nm} of each sample was measured. GlcN-6-P standards in cell lysate were determined in parallel to generate a standard curve.

Protein Assays

Protein concentrations were determined by the method of BRADFORD[21] using BioRad protein assay reagent and BSA fraction V as a standard.

Results and Discussion

A Novel Assay to Measure Ketol-isomerase Activity

Some methods to determine ketol-isomerase activity were previously reported, *i.e.* the Morgan-Elson assay[20], a spectrophotometric assay[22] and a high performance liquid chromatography (HPLC) assay[23]. But, none of them is suitable for high throughput screening. For example, boiling and addition of unstable reagents are needed in the Morgan-Elson assay, and measurement of ultra-violet absorption is needed in the spectrophotometric assay. Therefore, the establishment of a new ketol-isomerase assay, which is applicable for microtiter plates, would permit screening for new antifungal drugs.

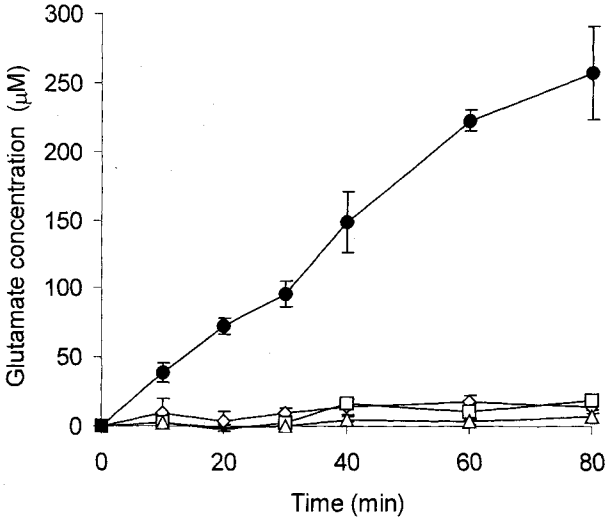
The experimental design of the new ketol-isomerase assay is summarized in Fig. 1. Glutamate, one of the products of the ketol-isomerase reaction, and β-NAD are converted to α-ketoglutarate and β-NADH by GLDH. Resulting β-NADH and exogenous PMS mediate the reduction of a tetrazolium salt, nitro BT[24]. The amount of formed nitro BT-formazan is measurable at OD_{585nm}. Accordingly, OD_{585nm} is proportional to the amount of glutamate formed by ketol-isomerase activity. To screen for ketol-isomerase inhibitors, crude cell lysate is incubated with substrates and test compounds, and the amount of glutamate produced by ketol-isomerase activity is determined by the addition of mixtures containing GLDH, β-NAD, PMS and nitro BT (nitro BT assay).

Using the nitro BT assay, we partially characterized ketol-isomerase activity present in crude cell lysates of *A. fumigatus*. The formation of glutamate by complete reactions was linear up to 60 minutes incubation at 37°C (Fig. 2). The specific activity of the lysate was 15 nmol/minute/mg protein. In contrast, in control mixtures lacking Fru-6-P, glutamine or cell lysate, the formation of glutamate was less than 8% of complete mixtures. These results indicate that glutamate was formed by ketol-isomerase activity, and not by other endogenous aminotransferase or deaminase activities.

In order to confirm that glutamate, detected by the nitro BT assay, was formed by ketol-isomerase activity, we compared the amounts of glutamate and GlcN-6-P formed by ketol-isomerase reactions using the nitro BT assay and the Morgan-Elson assay, respectively. These results are shown in Fig. 3. Note that there were no differences between the amounts of glutamate and GlcN-6-P formed in the reaction mixtures measured by these independent assays. These results clearly show that both glutamate and GlcN-6-P were formed by ketol-isomerase activity present in *A. fumigatus* crude lysates, and that the activity was successfully quantitated by the nitro BT assay. In addition, the assay was little influenced by other endogenous enzymes or compounds present in crude lysates.

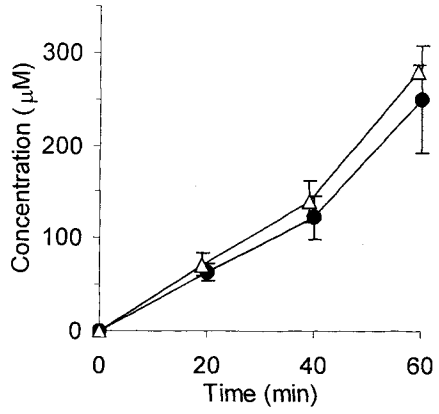
It is well known that eukaryotic ketol-isomerases are feedback-inhibited by UDP-GlcNAc[15,16,25~28]. Therefore,

Fig. 2. Ketol-isomerase activity of *A. fumigatus*.



Complete reaction mixture (●) containing 10 mM fructose-6-phosphate, 10 mM glutamine and 0.2 mg/ml *A. fumigatus* cell lysate, and control mixtures lacking fructose-6-phosphate (◇), glutamine (□) or lysate (△) were incubated at 37°C. The amount of glutamate was determined as described in Materials and Methods. The experiment was performed in triplicate. Each symbol represents the mean and the error bars represent the standard deviation.

Fig. 3. Glucosamine-6-phosphate and glutamate production by *A. fumigatus* ketol-isomerase activity.



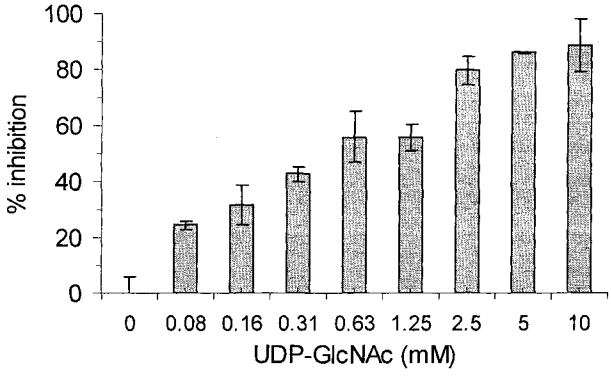
Each reaction mixture containing 10 mM fructose-6-phosphate, 10 mM glutamine and 0.3 mg/ml *A. fumigatus* cell lysate was prepared and incubated at 37°C for the indicated times as described in the Materials and Methods. The amount of glucosamine-6-phosphate (△) and glutamate (●) formed by each mixture was determined by the Morgan-Elson assay and the nitro BT assay, respectively, as described in Materials and Methods. The experiment was performed in triplicate. Each symbol represents the mean and the error bars represent the standard deviation.

using the nitro BT assay, we determined the effect of UDP-GlcNAc on *A. fumigatus* ketol-isomerase activity in crude lysates. The inhibitory effect of UDP-GlcNAc was dose-dependent and the IC_{50} was 0.48 mM (Fig. 4). The IC_{50} for *A. fumigatus* enzyme was comparable to those for *C. albicans*[26] and *Saccharomyces cerevisiae*[25] enzymes, but, was much higher than those for mouse, rat and human liver enzymes[27,28].

To date, ketol-isomerase activity has been partially characterized from some fungi and yeasts[14~18,25], but not from *A. fumigatus*. In preliminary studies, we determined that the highest specific activity of *A. fumigatus* ketol-isomerase was found using KI buffer. The activity in KI buffer was not degraded after freeze-thaw, and was stable at −80°C for at least 4 weeks (data not shown). These findings and the new assay will enable large scale screening for *A. fumigatus* ketol-isomerase inhibitors.

N-Acetylglucosamine is an important constituent not only for fungal cell wall chitin but also for bacterial cell wall peptidoglycan. BADET *et al.*[22] reported that the specific activity of *Escherichia coli* ketol-isomerase in crude

Fig. 4. Effect of UDP-*N*-acetylglucosamine on *A. fumigatus* ketol-isomerase activity.



The indicated amounts of UDP-GlcNAc were incubated with 10 mM fructose-6-phosphate, 10 mM glutamine and 0.3 mg/ml *A. fumigatus* cell lysate at 37°C for 60 minutes. The amount of glutamate formed by each mixture was determined as described in Materials and Methods. The experiment was performed in triplicate. Each bar represents the mean and the error bars represent the standard deviation.

Table 1. Screening of extremophilic bacterial extracts for ketol-isomerase inhibitors[a].

Antifungal activity[b]			Ketol-isomerase inhibition	
Candida spp.[c]	*A. fumigatus*	No. of extracts	+[d]	–
–	–	360	0	360
+	–	14	0	14
–	+	0	0	0
+	+	12	1 (MB365)	11

[a] Samples were extracted from the spent medium of extremophilic bacteria with ethyl acetate, dried and resuspended in DMSO[29]. Extracts were tested for inhibition of fungal cell growth and *A. fumigatus* ketol-isomerase activity.
[b] Antifungal activity was initially determined using an agar diffusion method. The activity of positives was confirmed by a microbroth dilution method. The samples that inhibited growth at ≦1000 μg/mL were judged as positive (+).
[c] *C. albicans* and *C. glabrata*
[d] (+) indicates >50% inhibition of enzyme activity using the nitro BT assay at 800 μg/mL; (–) indicates no significant inhibition.

extracts, prepared by grinding with alumina, was 19 nmol/minute/mg, similar to that found for *A. fumigatus*. We suggest that bacterial ketol-isomerase activity in crude bacterial lysates may be determined using the nitro BT assay, *i.e.*, the nitro BT assay may be applicable to screen for both fungal and bacterial ketol-isomerase inhibitors using crude cell lysates from each organism.

A Pilot Scale Screening for *A. fumigatus* Ketol-isomerase Inhibitors

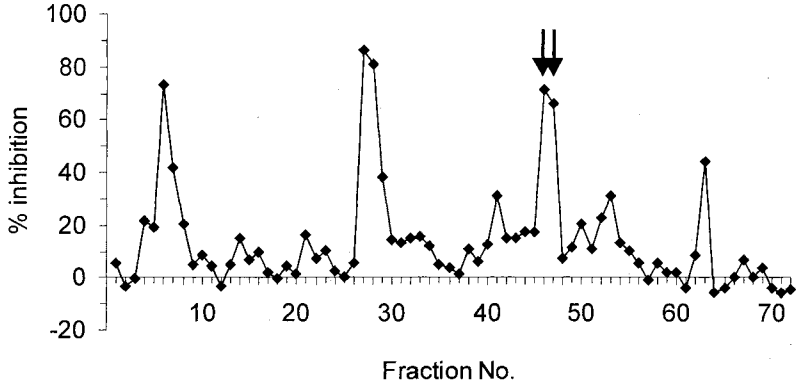
A major difficulty with *in vitro* enzyme screening is that the number of samples that test positive is often in excess of 5%, indicating that the screening is not selective enough to be useful. To determine if this were the case for the nitro BT assay that we developed, we performed a pilot screening with 386 ethyl acetate extracts of spent media from extremophilic bacterial cultures (Montana Biotech, Belgrade, MT) that were part of another screening program for antifungals[29]. Antifungal activity was determined by either microbroth dilution or agar diffusion methods[30]. A summary of these results is presented in Table 1. Fourteen samples inhibited the cell growth of *Candida* spp. (*C. albicans* ATCC 90028 and *C. glabrata* ATCC 48435), and 12 samples inhibited the growth of both *Candida* spp. and *A. fumigatus* ATCC 16424. On the other hand, only one sample (MB365) inhibited *A. fumigatus* ketol-isomerase activity. Interestingly, MB365 also inhibited the growth of all tested organisms (IC_{50}: 1000 μg/ml for *C. albicans*, 500 μg/ml for *C. glabrata*, 62.5 μg/ml for *A. fumigatus*, IC_{90}: 1000 μg/ml for *C. albicans* and *C. glabrata*, 500 μg/ml for *A. fumigatus*,). In contrast, the inhibitory effect on ketol-isomerase activity was 76% at 400 μg/ml and 30% at 100 μg/ml (data not shown).

Screening natural products using fungal whole cell assays to identify antifungal compounds often selects compounds that have no specificity to fungi. Using the *in vitro* ketol-isomerase assay, we found less than 1% positives in the pilot scale screening. Importantly, the positive sample showed antifungal activity. Although the number of positives detected by the nitro BT assay were much less than that detected by the whole-cell assays, the enzyme assay will be more efficient for screening for selective antifungal drugs compared to whole-cell assays.

Partial Purification of a Ketol-isomerase Inhibitor

Based on the results described above, we chose the extract from MB365 as a subject for further studies. The sample was separated into 72 fractions by reverse phase high performance liquid chromatography using methodology to be described elsewhere (O'ROURKE, *et al.*, in preparation). The effect of each fraction on *A. fumigatus* ketol-isomerase activity was determined using the nitro BT assay. In addition, antifungal activity of each fraction against *C. glabrata* ATCC 48435 was also determined

Fig. 5. Effect of MB365 fractions on *A. fumigatus* ketol-isomerase activity.



The extract of MB365 was fractionated by reverse phase HPLC. Each fraction was dried and dissolved in DMSO. The effect on *A. fumigatus* ketol-isomerase was determined by the nitro BT assay as described in Materials and Methods.

The antifungal activity was determined by an agar diffusion method using *C. glabrata* as a test organism. The arrows indicate fractions active against *C. glabrata* (inhibitory zone diameter; ≧10 mm).

using an agar diffusion method. These results are shown in Fig. 5. There were four peaks that inhibited the enzyme activity. The first active peak corresponds to fraction 6, and the second and the third peaks correspond to fractions 27~28 and 46~47, respectively. The inhibitory effect in the fourth peak (fraction 62) was slightly lower than that in the other three peaks. Interestingly, antifungal activity was found only in the third peak (fractions 46~47).

To investigate the reason why fractions 6, 27, and 28 had no antifungal activity but resulted in a decrease in OD_{585nm}, we tested the effects of the fractions on ketol-isomerase activity using the Morgan-Elson assay. Although inhibition was found in fractions 6, 27, and 28 by the nitro BT assay, these fractions showed no effect on the formation of GlcNAc-6-P as determined by the Morgan-Elson assay (data not shown). In the nitro BT assay, an exogenous enzyme, GLDH, needs to be added. Therefore, GLDH inhibitors as well as ketol-isomerase inhibitors could be detected using the nitro BT assay. To confirm this hypothesis, we tested the inhibitory effect of each fraction on GLDH activity by using a modification of the nitro BT assay. The procedure was identical to the nitro BT assay (described in Materials and Methods) except that glutamate dissolved in KI buffer was added instead of crude lysate and substrates. Fractions 6, 27, and 28 inhibited GLDH activity and the inhibitory effects were comparable to those determined by the nitro BT assay (data not shown). Taken together, we conclude that these three fractions have no inhibitory effect on ketol-isomerase activity but inhibited GLDH activity.

In contrast, fractions 46 and 47 had no effects on GLDH activity (data not shown). It is clear that these two fractions inhibited *A. fumigatus* ketol-isomerase activity but not the assay to detect the formation of glutamate. NMR and MS analyses to determine the identity of the active compound are in progress. The structural elucidation and the biological activity will be the subjects of a subsequent manuscript.

Conclusion

We have established a new assay for ketol-isomerase activity using *A. fumigatus* crude lysate. The assay is specific and can be used easily for high throughput screening for new antifungal drugs. We tested 386 extracts of extremophiles as a pilot scale screening and found an active extract. The extract also inhibited fungal cell growth. This assay will lead to the isolation of entirely new classes of antifungal drugs with a novel mechanism of action. In addition, this assay will be also applicable for screening for new antibacterial drugs.

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